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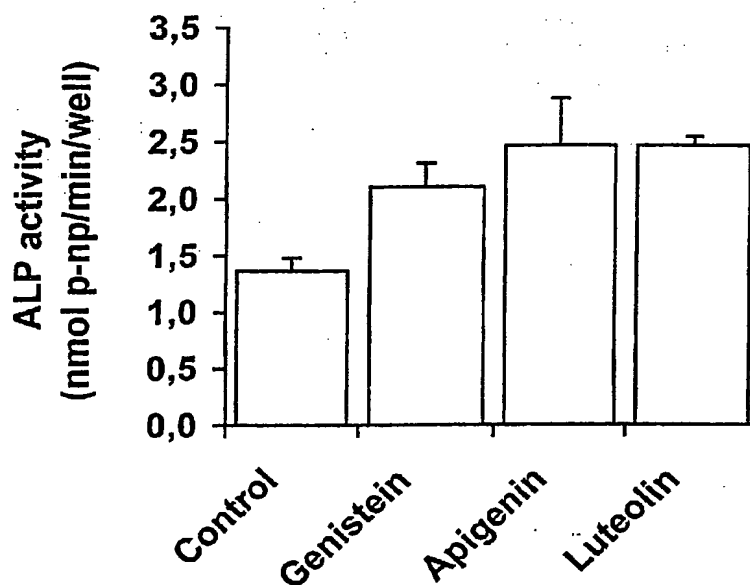
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(54) Title: **PREPARATION FOR THE PREVENTION AND/OR TREATMENT OF ALTERED BONE METABOLISM**



(57) Abstract: The present invention relates to the use of one or more of apigenin, luteolin and morin and functional analogues thereof for the manufacture of a preparation for the treatment and/or prevention of a disorder related to altered bone metabolism, in particular osteoporosis, osteopenia, Paget's disease, osteolytic metastasis in cancer patients, osteodystrophy in liver disease or the altered bone metabolism caused by renal failure or haemodialysis, bone fracture, bone surgery, pregnancy, anorexia nervosa, and in immobile, malnourished and weight losing persons.



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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Preparation for the prevention and/or treatment of altered bone metabolism

5 The present invention relates to a preparation for the prevention and/or treatment of disorders related to altered bone metabolism.

Although it looks like an inert mineral substance, bone is a living, dynamic connective tissue that is constantly being renewed. It contains cells and specialized collagen fibers, encrusted
10 with a crystalline mineral. Together, the minerals, cells, fibers and ground substance form the organic matrix or "osteoid". Bone mineral is a complex salt of calcium phosphate, citrate, boron, sodium, zinc, magnesium and fluorine. Most bones in the skeleton contain two types of bone tissue: compact bone and trabecular bone. Bone is constantly turned over by a combination of bone formation by osteoblasts and bone resorption by osteoclasts. If blood
15 calcium levels are lower, resorption of the bone increases to fulfill calcium requirements elsewhere in the body.

Altered bone metabolism can be characterized by a misbalance between bone formation and bone resorption. It can occur in relation to several types of disorders. Examples are
20 osteoporosis, osteopenia, Paget's disease, osteolytic metastasis in cancer patients, osteodistrophy in liver disease and the altered bone metabolism caused by renal failure or haemodialysis, bone fracture, bone surgery, pregnancy, anorexia nervosa and in immobile, malnourished and weight losing persons.

25 The major bone disease in the older population is osteoporosis. This disease is characterized by extensive bone loss leading to an increase in bone fragility and a greater liability for fractures. It causes considerable pain, disability, disfigurement and loss of independence, and is a cost and burden to health services. Internationally, more than 1.5 million fractures occur every year as a result of osteoporosis.

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There are two types of osteoporosis recognized. The first typically occurs between the ages of 50 and 75 and affects six times as many women (post-menopausal osteoporosis) than men.

Bone loss is accelerated with aging. Most fractures occur in trabecular bone, especially the wrist and spinal vertebrae. The second type is referred to as senile osteoporosis and affects both men and women over 75 years of age and does not involve greater than normal bone loss. The risk factors for both types of osteoporosis are high caffeine intake, alcohol consumption, low body weight and low calcium intake.

The most prominent and well documented cause of post-menopausal osteoporosis is estrogen deficiency. After the menopause, the ovaries cease to produce this hormone, which directly relates to loss of bone mineral content. A very effective treatment for post-menopausal symptoms, including osteoporosis, is hormone replacement therapy (HRT), and this estrogen replacement effectively prevents the development of osteoporosis. Because HRT may have serious side effects, e.g. breast tissue growth stimulation, nutritional supplements for the prevention of osteoporosis are gaining popularity.

For other types of osteoporosis, the main cause has not been defined yet. Since elderly people usually have low amounts of calcium in their diet, their body calcium levels are low. The body, however, needs a stable blood calcium level for several other important functions, such as cell signaling, and thus bone resorption is increased to maintain adequate levels of this mineral in case of insufficient calcium intake.

The common approach to reduce bone loss with nutritional supplements is the use of phytoestrogens. These compounds are derived from botanical sources, such as soy or black cohosh, and have a molecular structure that resembles estrogen. The phytoestrogens can be classified as isoflavons (genistein, daidzein), lignans and coumestans. The mechanism of action of phytoestrogens has not been resolved yet. It is possible that their activity is related to the fact that they bind to the estrogen receptors in a similar way as estrogen, leading to comparable effects as conventional HRT. It is also possible that they directly act on osteoclasts and osteoblasts via an estrogen receptor independent mechanism.

Phytoestrogens and in particular isoflavones are for instance described in Alekel, D.L. et al, 2000, "Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women", Am J Clin Nutr. 72:844-52; Messina, M. and Messina V., 2000,

"Soyfoods, soybean isoflavones, and bone health: a brief overview", J Ren Nutr. 10:63-8;
Erdman J.W. et al, 2000, "Provocative relation between soy and bone maintenance.", Am J
Clin Nutr. 2000; 72:679-680.

- 5 In WO 98/50054 it is described that vegetable extracts can be used for the treatment of
diseases or conditions characterized by increased bone resorption. Examples described are
allium (onion, garlic), petroselinum (parsley), brassica (broccoli) and eruca (arugula or
roquette) extracts. In WO 00/20014 extracts of lycopersicon (tomato) are described. In WO
00/20015 extracts of anethum (dill) are mentioned.

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It has now been found that a specific group of natural components can be used for the
treatment and/or prevention of the above mentioned disorders related to altered bone
metabolism. The present invention thus provides the use of one or more members selected
from the group consisting of apigenin, luteolin, morin and functional analogues thereof for the
15 manufacture of a preparation for the treatment and/or prevention of a disorder related to
altered bone metabolism.

15

The components of the invention (apigenin, luteolin and morin) are preferably obtained from
natural sources. Also mixtures thereof can be used. Further suitable are functional analogues
20 such as glycosylated forms and plant extracts containing these components. The components
according to the invention are used in a total amount of 0.1 mg to 1 g, preferably 5 to 700 mg,
more preferably 25 to 500 mg per daily dose.

20

The preparation for the treatment and/or prevention of a disorder related to altered bone
25 metabolism according to the present invention can be a pharmaceutical preparation, such as a
tablet, a capsule, a sachet or a suppository or a nutritional preparation, such as a nutritional
supplement, a tube feeding, a complete food or a health promoting preparation. The
preparation is suitable for the treatment of these disorders in mammals including humans.

25

30 The preparations preferably also contain one or more of the following:

- a. calcium;
- b. bone minerals, such as magnesium and boron;

30

- c. gamma linolenic acid;
- d. vitamins such as vitamin D and vitamin K;
- e. estrogens or one or more estrogen mimicking compounds used in Estrogen Replacement Therapy;
- 5 f. biphosphonates and
- g. isoflavones.

Calcium is added to increase calcium deposition in bones. The calcium source can be any suitable inorganic or organic compound containing calcium. Examples are inorganic calcium salts, for example calcium chloride, calcium phosphate, calcium sulphate, calcium oxide, 10 calcium hydroxide or calcium carbonate. Examples of organic calcium compounds are milk powder or calcium caseinate, calcium citrate, calcium malate, calcium citrate malate or calcium lactate. The amount of calcium is preferably 200 to 1500 mg per daily dose.

15 Bone minerals are added to ensure strong bones. Preferably the preparation contains 100 mg to 500 mg magnesium and 2 mg to 6 mg boron per daily dose.

Gamma linolenic acid is used to regulate calcium metabolism, preferably in an amount of 25 mg to 100 mg per daily dose.

20 Vitamins are added as cofactors for optimal bone metabolism. Preferably, daily vitamin K dose should be 25 µg to 5 mg vitamin K. Vitamin D is used to increase calcium uptake from the gut. Preferably 5 µg to 20 µg (200 IU to 800 IU) per daily dose is present in the preparation.

25 Estrogens or one or more estrogen mimicking compounds used in Estrogen Replacement can also be included in the preparation for the treatment and/or prevention of osteoporosis. Examples of these compounds are phytoestrogens, like genistein, lignans or coumerans or pharmaceutical preparations like 17β-estradiol, esterified estrogens, estrone sulfate, 30 conjugated equine estrogen, and ethinylestradiol. For the phytoestrogens the amount of these compounds is 5-100 mg. For the pharmaceutical preparations the active amount is defined by the instructions of the manufactures.

One or more biphosphonates are used to inhibit the osteoclastic bone resorption. Examples of these compounds are alendronate and risedronate. Preferably the amount of these compounds is 5 mg to 50 mg.

Isoflavones can be obtained (isolated) from soy or black cohosh or can be synthetic isoflavones. Isoflavones can be added in an amount of 10 to 75 mg per daily dose.

The preparations of the invention can further contain other sources of energy, such as fats and carbohydrates, proteins, vitamins, minerals, fibers, flavors, preservatives, colorants, sweeteners, etc.

The active components according to the invention can be used as pure substances but can also be in the form of extracts of plants or parts thereof. Morin can for example be used in the form of extracts of jackfruit (*Artocarpus heterophyllus*) and white mulberry (*Morus alba*). Luteolin can for example be used in the form of extracts of peanut shells (*Arachis hypogaea*), artichock (*Cynara scolymus*) and celery (*Apium graveolens*). Apigenin can for example be used in the form of extracts of chamomillae (*Matricaria recutita*) or celery (*Apium graveolens*).

Extracts can be used by methods known in the art, e.g. by extracting the starting material with water or a food grade solvent, or mixtures thereof. A preferred food grade solvent is ethanol. After extraction the liquid phase containing the active ingredients can be concentrated or dried by evaporation or freeze drying.

Extracts of petroselinum (parsley), eruca (arrugula or roquette), lycopersicon (tomato) and anethum (dill) are excluded from the invention.

The components and preparations of the present invention can in particular be used for disorders characterized by altered bone metabolism. The disorder can be osteoporosis, osteopenia, Paget's disease, osteolytic metastasis in cancer patients, osteodistrophy in liver disease or the altered bone metabolism caused by renal failure or haemodialysis, bone

fracture, bone surgery, pregnancy, anorexia nervosa, and in immobile, malnourished and weight losing persons.

5 The invention will now be illustrated by means of the following examples and the attached figure 1 which shows the alkaline phosphatase activity in mouse bone marrow cells after 3 weeks exposure to the components of the invention.

Examples

1. Formulations

10 Nutritional supplement 1

Components	Amount per serving	
Apigenin	15	mg
Luteolin	15	mg

Nutritional supplement 2:

Components	Amount per serving	
Morin	10	mg
Apigenin	20	mg
Luteolin	5	mg

15 Nutritional supplement 3:

Components	Amount per serving	
Calcium	800	mg
White Mulberry extract (providing glycosylated morin)	10	mg
Chamomilla extract (providing glycosylated apigenin)	10	mg
Artischock extract (providing glycosylated luteolin)	10	mg
<i>Amounts of active components are expressed as aglycons</i>		

7.

Nutritional supplement 4:

Components	Amount per serving	
Calcium	800	mg
Vitamin D3	200	IU
Morin	10	mg
Apigenin	10	mg
Luteolin	100	mg

Nutritional supplement 5:

Components	Amount per serving	
Calcium as Calcimate®	800	mg
Vitamin D3	200	IU
Magnesium	200	mg
Boron	2	mg
Morin	5	mg
Apigenin	25	mg
Luteolin	20	mg
Gamma linolenic acid	25	mg

5 Nutritional supplement 6:

Components	Amount per serving	
Calcium as Calcimate®	800	mg
Vitamin D3	200	IU
Magnesium	200	mg
Boron	2	mg
Isoflavones	30	mg
Apigenin	50	mg
Gamma linolenic acid	25	mg
Vitamin K1	500	µg

Nutritional supplement 7:

Components	Amount per serving	
Calcium	800	mg
Vitamin D3	200	IU
Black cohosh providing isoflavones	40	mg
Soy extract providing glycosylated isoflavones	30	mg
Morin	10	mg
Apigenin-7-glucoside	10	mg
Luteolin-4' glucoside	10	mg
<i>Amounts of active components and isoflavones are expressed as aglycons</i>		

Stimulating effect on mouse bone marrow cells

- 5 The following experiment is illustrated in Figure 1, which shows the alkaline phosphatase activity in mouse bone marrow cells after 3 weeks exposure to the components of the invention.

Experiment

- 10 Bone marrow cells were isolated from six months old female NMRI mice. After washing, the cells were diluted in phenol red free α -MEM (1% p/s, 15% charcoal treated FCS) and seeded in 24-well plates (1.25×10^6 cells/well). After 24 hours incubation (37°C, 5% CO₂) the cells were exposed to the active components (1×10^{-5} , 1×10^{-6} and 1×10^{-7} M) together with ascorbic acid (50 μ g/ml) and β -glycerolphosphate (1 mM). Exposure media were refreshed every 3
- 15 days.

After 2 and 3 weeks of incubation, cells were washed with PBS and stored at -20°C. After lysis DNA amounts and alkaline phosphatase activities were determined as described below. The DNA amounts are measured to determine to the number of cells and the alkaline phosphatase activity is used as a marker for osteoblast differentiation.

20

Lysis

300 μ l lysis buffer (1 x SSC/0.01% SDS) was added to the frozen cells. After 1 hour incubation (37°C) the plates were sonified for 10 minutes and cell suspensions were used for ALP activity measurements.

Alkaline phosphatase (ALP) activity assay

25 μ l cell lysate was transferred in duplicate to 96-well plates. 25 μ l of 1*SSC/0.01%SDS served as a negative control. 175 μ l of ALP-assay buffer (10 mM glycine, 0.1 mM MgCl_2 , 0.01 mM ZnCl_2 , 6 mM p-nitrophenylphosphate, pH 10.5), was added to every well. ALP activity was measured at a wavelength of 405 nm at room temperature during 10 minutes at 30-second intervals. The results of this assay are shown in figure 1, which shows the alkaline phosphatase activity in mouse bone marrow cells after 3 weeks exposure to the active components. Activity is expressed as nmol p-nitrophenol production/minute/well. (Mean \pm sem, 2 independent experiments measured in duplicate)

Figure 1 shows that incubations of mice bone marrow cells with 1 mM apigenin and luteolin result in an increased alkaline phosphatase signal in differentiated osteoblasts. The stimulation is more pronounced than the effect of the isoflavone genistein of which bone loss prevention has been shown *in vivo* (rat and human). Similar results were obtained with morin (data not shown).

Claims

1. Use of one or members selected from the group consisting of apigenin, luteolin, morin and functional analogues thereof for the manufacture of a preparation for the treatment and/or prevention of a disorder related to altered bone metabolism in a mammal.
2. Use according to claim 1, wherein the disorder is osteoporosis, osteopenia, Paget's disease, osteolytic metastasis in cancer patients, osteodystrophy in liver disease or the altered bone metabolism caused by renal failure or haemodialysis, bone fracture, bone surgery, pregnancy, anorexia nervosa, and in immobile, malnourished and weight losing persons.
3. Use according to claim 1 or 2, wherein the preparation contains one or more extracts selected from the group consisting of jackfruit (*Artocarpus heterophyllus*), white mulberry (*Morus alba*), peanut shells (*Arachis hypogaea*), artichock (*Cynara scolymus*), celery (*Apium graveolens*) and chamomillae (*Matricaria recutita*).
4. Use according to any of the preceding claims, wherein the preparation is a pharmaceutical preparation, such as a tablet, a capsule, a sachet or a suppository, or a nutritional preparation, such as a nutritional supplement, a tube feeding, a complete food or a health promoting preparation.
5. Use according to any of the preceding claims, in combination with isoflavones, calcium, vitamin D and/or vitamin K.
6. Preparation containing
 - a. apigenin, luteolin, morin and/or functional analogues thereof and
 - b. a second ingredient providing an effective amount of isoflavones, calcium, vitamin D and/or vitamin K.
7. Preparation according to claim 6, wherein the isoflavones are isolated from soy or black cohosh.

8. Preparation according to claim 6 or 7, which contains extracts selected from the group consisting of jackfruit (*Artocarpus heterophyllus*), white mulberry (*Morus alba*), peanut shells (*Arachis hypogaea*), artichock (*Cynara scolymus*), celery (*Apium graveolens*) and chamomillae (*Matricaria recutita*).

5

9. Preparation according to any of claims 6 to 8, further containing one or members selected from the group consisting of magnesium, boron and gamma linolenic acid.

10. Preparation according to any of claims 6 to 9, further containing estrogens or one or more estrogen mimicking compounds used in Estrogen Replacement Therapy.

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11. Preparation according to any of claims 6 to 10, further containing one or more biphosphonates.

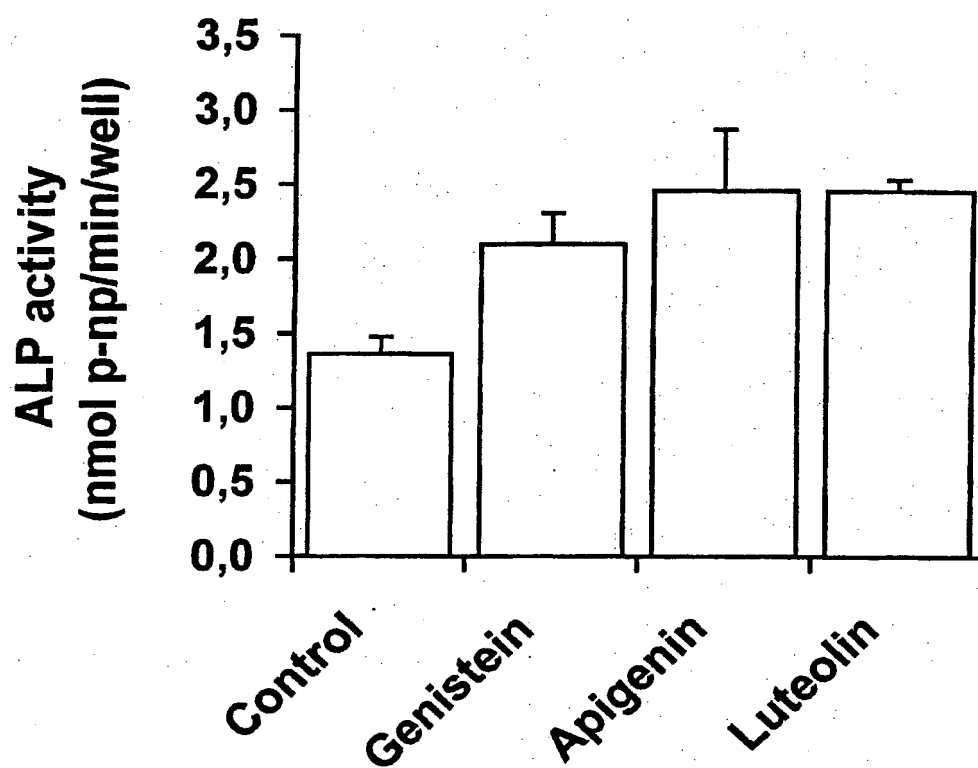
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12. Use of a preparation according to any of claims 6 to 11 for the treatment and/or prevention of a disorder related to altered bone metabolism.

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13. Use according to claim 12, wherein the disorder is osteoporosis, osteopenia, Paget's disease, osteolytic metastasis in cancer patients, osteodistrophy in liver disease or the altered bone metabolism caused by renal failure or haemodialysis, bone fracture, bone surgery, pregnancy, anorexia nervosa, and in immobile, malnourished and weight losing persons

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A23L1/30 A61K35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 01 03687 A (CEDARS SINAI MEDICAL CENTER) 18 January 2001 (2001-01-18) page 4, line 28 -page 5, line 19 page 7, line 28 - line 35 page 9, line 25 -page 10, line 35 page 12, line 5 - line 37 page 15, line 7-15; claims 1,7,9,10,12,14,23,25,26,55-57,59,60 ---	1,2,4-7, 10,12,13 3,8,11
X Y	WO 91 11117 A (UNIV TEXAS) 8 August 1991 (1991-08-08) page 11, line 4 - line 22 page 20, line 26 -page 21, line 6 page 24, line 15 - line 25 page 25, line 4 -page 26, line 28 page 30, line 10 - line 20; claims 1-3,18,30,34 --- -/-	1,2,4-6, 9,12,13 3,8,11

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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